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ORIGINAL PAPER

Sample clean-up methods, immunoaffinity chromatography and solid phase extraction, for determination of deoxynivalenol and deepoxy deoxynivalenol in swine serum

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Abstract Concentrations of deoxynivalenol (DON) and deepoxy deoxynivalenol (DOM-1) in animal blood are important parameters for studies in toxicology and biological detoxification of DON. Clean-up methods, using either immunoaffinity chromatography (IAC) or solid phase extraction (SPE), were compared in order to determine the free form of DON or DOM-1 and the sum amount (free form plus glucuronide conjugated form of DON or DOM-1), respectively, in swine serum. Detection was achieved by high performance liquid chromatography with ultraviolet detection (HPLC-UV). Compared with the SPE-HPLC method, the IAC-HPLC method provided lower quantitation limit (DON: 18 vs 42 ng/ml; DOM-1: 21 vs 30 ng/ml) and higher recoveries (DON: 93.4–102.7% vs 63.7–85.3%; DOM-1: 85.5–91.1% vs 68.0–82.6%). Compared with previously published methods, the developed IAC-HPLC method removed analytical interferences from swine serum in one quick and easy step, and eliminated steps of extraction with organic solvent and/or pre-purification using SPE cartridges. This IAC-HPLC method was used to analyze swine serum samples collected from pigs that were evaluated in a feeding trial of a microbiological detoxification of DON. No DON or DOM-1 were detected in serum samples from pigs given a toxin-free diet or a microbial control diet. In serum samples from pigs given a DON diet (5 mg/kg of DON), free form DON and sum free DON + conjugated DON were 38.8±13.7 and 49.8±14.1 ng/ml, respectively. In serum samples from those given a detoxified-DON diet (DON was transformed to DOM-1), free form DOM-1 was detected but not quantified, and the sum DOM-1 was found as 47.5±6.3 ng/ml.

Keywords Deoxynivalenol (DON) · Deepoxy-deoxynivalenol (DOM-1) · DON glucuronide conjugate · DOM-1 glucuronide conjugate · Swine serum · Immunoaffinity column

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Introduction

Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium* spp. (Nelson 2002). DON is found worldwide in cereal grains and in animal feed and human food produced from contaminated grains, which creates a food safety risk. The toxicity of DON is largely due to its ability to inhibit protein, DNA and RNA synthesis, thus DON has immunosuppressive effects to human and animals (Pestka and Smolinski 2005; Pestka 2007). Physical, chemical and biological detoxification techniques are being evaluated for their usefulness in transforming DON into other, less toxic, compounds for improved safety in food and feed chains. Many toxicology studies have been conducted for evaluation of the effects of DON and its detoxification metabolites on feed intake and growth performance of swine. Deepoxy deoxynivalenol (DOM-1) can be found in swine as a metabolite (Döll et al. 2003, 2007; Eriksen et al. 2003; Dänicke et al. 2005; Goyarts and Dänicke 2006). In addition, DON and DOM-1 glucuronide conjugates have been found as DON and DOM-1 metabolites (Eriksen et al. 2003; Dänicke et al. 2005; Goyarts and Dänicke 2006; Seeling et al. 2006; Wu et al. 2007). These conjugated

forms are important for studies in toxicokinetics, tests of detoxifying agents, and other related studies. Therefore, the concentrations of the sums of free and conjugated forms of DON and DOM-1 in swine blood are also important parameters of blood chemistry in the evaluation of the toxicity of this toxin and its detoxification product.

Currently, there is no official method for determination of DON in animal blood or other tissues available. Gas chromatography (GC), high performance liquid chromatography-mass spectrometry (HPLC-MS) and HPLC-ultraviolet detection (HPLC-UV) have been applied to analysis of trichothecenes in animal tissues (Eriksen et al. 2003; Fuchs et al. 2002; Valenta et al. 2003; Bily et al. 2004). The challenges of determination of DON and DOM-1 in animal blood are their low concentration and interference of other (macro- and/or micro-) chemicals in the blood complex. Thus, the clean-up methods are critical for the analysis. To analyze DON in plasma, multiple steps including pre-purification using a C_{18} cartridge, partition with toluene:ethyl acetate (9:1, v/v), purification using a Florisil column, and concentration by removal of organic solvents were applied before GC analysis (Swanson et al. 1982; Cote et al. 1985). Extraction with acetonitrile and clean-up using an alumina-charcoal column was used to detect DON in swine plasma (Prelusky and Trenholm 1991). Extraction with ethyl acetate was applied to detect nivalenol, 3-acetyl-DON, DON and DOM-1 in swine plasma (Hedman et al. 1997; Eriksen et al. 2003). Most recently, clean-up with immunoaffinity chromatography (IAC) has been commonly used in analyses of DON (Janes and Schuster 2001), and both DON and DOM-1 (Valenta et al. 2003) in blood, and gave lower limits of detection and higher recoveries compared to above methods. These two methods are also appropriate for more difficult matrices like urine (Janes and Schuster 2001; Valenta et al. 2003) and bile (Dänicke et al. 2005). However, Janes and Schuster's method (2001) still required extraction with organic solvent acetonitrile before loaded on IAC, and Valenta et al.'s method (2003) needed a pre-purification with a ChemElut column before loaded on IAC, and generated large amounts of organic solvent wastage (35 ml acetyl acetate for each 1.5 ml serum) when eluted DON or DOM-1 from the ChemElut column. Therefore, simple and effective IAC clean-up methods are needed for low detection limit and high recovery.

In addition to the IAC method, solid phase extraction (SPE) is used to selectively extract, concentrate, and purify target analytes prior to analysis. It is one of the most popular extraction methods used in routine laboratory analysis (Young et al. 2007). A SPE method without further purification and/or extraction with organic solvents has not been reported for analysis of DON or DOM-1 in swine serum. This present work aims to (1) develop a simplified IAC method and a new SPE method; (2) evaluate these two clean-up methods; and (3) select the superior method between these two for more efficient determination of DON and DOM-1 in swine blood by HPLC-UV.

Materials and methods

Chemicals

DON standard and β-glucuronidase (type H-2 from *Helix pomatia*) were purchased from Sigma–Aldrich (Oakville, ON, Canada). The enzyme β-glucuronidase was dissolved in 0.2 mol/l sodium acetate buffer (pH 5.5) to make a 30,000 units/ml solution. DOM-1 was purified using a semi-preparative HPLC with a Luna C18 (150×21.2 mm, 5 μm) column (Phenomenex; Torrance, CA, USA) from an extract of a microbial culture, in which DON was transformed to DOM-1 (Young et al. 2007). HPLC grade solvents (acetonitrile and methanol) were obtained from Caledon Laboratories (Georgetown, ON, Canada).

Blood sample collection

Swine blood was drawn from the retro-orbital sinus after the noon feed, collected in tubes without anticoagulant, centrifuged at 1,500 *g* for 15 min at 4°C, and the serum was then frozen at −80°C before analysis. Spiked swine serum sample were made from clean serum (1.5 ml) by adding DON (1,000 ng/ml, 0.075 ml) and DOM-1 (1,000 ng/ml, 0.075 ml) to make final concentrations of DON and DOM-1 at 50 ng/ml.

Clean-up using IAC

DONPREP® IAC columns (R-Biopharm Rhône, Glasgow, UK) were used in this study. For the samples tested for free form DON and DOM-1, 1.5 ml distilled water was added to each 1.5 ml serum. The whole diluted serum sample (3.0 ml) was applied to an IAC and allowed to pass through the column. For the samples tested for total DON and DOM-1, 1.0 ml 0.2 mol/l sodium acetate buffer (pH 5.5) and 0.5 ml 30,000 units/ml β-glucuronidase solution (no enzyme for control) were added to each 1.5 ml serum. The reaction mixture was incubated at 37°C in a water bath overnight (Wu et al. 2007). The reacted serum sample (3.0 ml) was applied to an IAC and allowed to pass through the column. The column was washed twice, with 5 ml distilled water each time. Methanol (1.5 ml) was pipetted to the column to elute DON or DOM-1 from the IAC. A flow rate of 1 ml/min was used in the whole clean-up procedure. The eluate was collected, dried under a gentle nitrogen stream, reconstituted in

0.2 ml 50% methanol aqueous solution and analyzed by HPLC. Injection volume was 100 µl.

Clean-up using solid phase extraction (SPE)

Strata impact protein precipitation (2 ml square well filter plate, 96-well plate) and Strata NH2 SPE cartridges (200 mg/3 ml) (Phenomenex) were used. To each of the three empty wells in the protein precipitation plate, 1.5 ml acetonitrile was added, followed by 0.5 ml swine serum containing DON and DOM-1 (50 ng/ml). The protein precipitation plate was agitated at room temperature (23°C) on a platform shaker at 100*g* for 20 min allowing protein to be precipitated. The plate was then placed in a Strata 96-well plate manifold, and the extracts from the three wells were collected and combined. The combined extract was applied on a Strata NH2 SPE cartridge that was pre-conditioned with 75% acetonitrile aqueous solution, and allowed to pass through the cartridge at a flow rate of 1 ml/min. The eluate was collected, dried under a gentle nitrogen stream, reconstituted in 0.2 ml 50% methanol aqueous solution and analyzed by HPLC. Injection volume was 100 µl.

HPLC analysis

Quantification and identification of DON and DOM-1 were achieved by using an Agilent Technologies 1100 Series HPLC system with a diode array detector (DAD) and a Phenomenex® Luna C18 (2) column (150 mm×4.6 mm, 5 µm). The binary mobile phase consisted of solvent A (methanol) and solvent B (water) and the gradient program began at 22% A, increased linearly to 41% A at 5 min, 100% A at 7 min, held 100% A from 7 to 9 min, and returned to 22% A at 11 min. There was a 2-min post-run under starting conditions for re-conditioning (He et al. 2007). The flow rate was 1.0 ml/min and the DAD detector was set at 218 nm. Identification of DON and DOM-1 was achieved by comparing their retention times and UV-Vis spectra with those of DON and DOM-1 standards. Quantification was based on reference to a calibration curve of DON and DOM-1 standard. Five concentrations of DON or DOM-1 (100, 250, 500, 750, 1,000 ng/ml) were measured in triplicate to produce a calibration curve.

Results and discussion

Linearity of DON and DOM-1

Instrument linearity for DON and DOM-1 was demonstrated using five standard solutions with concentrations from 100 to 1,000 ng/ml. Linear regression correlation coefficients (R^2) of DON and DOM-1 were 0.9992 and 0.9989, respectively. Standard solutions of DON or DOM-1 of 100 ng/ml were the ones producing a peak with a signal/noise ratio of 10 (S/N=10) for both DON and DOM-1 in this HPLC instrument.

Comparison of IAC and SPE methods

IAC, a separation technique based on antibody affinity, is specific to target analyte and with minimum interference of co-extracted substances. This technique has been widely used in analysis of DON in various matrixes (Cahill et al. 1999; Valenta and Dänicke 2005; Lattanzio et al. 2007). SPE extraction has also been developed as a conventional method for sample preparation for DON analysis (Swanson et al. 1982; Cote et al. 1985; Prelusky and Trenholm 1991). However, currently, there is no SPE method applied to clean-up serum samples without further purification and/or extraction with organic solvents. In this study, a clean-up method was developed by combining impact protein precipitation and SPE for purification of DON and DOM-1 from the biological mass of swine serum.

Method detection limit (MDL) is used to study the theoretical detection capability of the method. The MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. MDL was computed as $\mathrm{MDL} = \mathrm{t}_{(\mathrm{n}-1,\,1-\alpha=0.99)} \times \mathrm{SD}$, where $t_{(\mathrm{n}-1,\,1-\alpha=0.99)}$ stands for the Students' t value appropriate for a 99% confidence level and a standard deviation estimate with $n-1$ degrees of freedom; SD stands for the standard deviation of the n replicates (Glaser et al. 1981; Federal Register 1995). Method quantitation limit (MQL) is three times of the MDL. Eight spiked swine serum samples (DON and DOM-1 final concentrations were 50 ng/ml) were analyzed for DON and DOM-1. A comparison of the IAC-HPLC and SPE-HPLC methods in MDL, MQL and recovery is summarized in Table 1. The MDLs and MQLs of the IAC-HPLC method for DON and DOM-1 were 2.3 and 1.4 times lower than those of the SPE method, respectively.

Recoveries of DON and DOM-1 in IAC-HPLC method ranged from 93.4 to 102.7% and from 85.5 to 91.1%, respectively. Recoveries of DON and DOM-1 in SPE-HPLC method ranged from 63.7 to 85.3% and from 68.0 to 82.6%, respectively, which are lower than those of the IAC method. It was also noticeable that there was a positive correlation between the recoveries in the SPE method with the concentrations of analytes in spiked samples, which implied that the SPE-HPLC method may perform better for samples with high concentrations of DON or DOM-1 (Table 1).

Table 1 Comparison of IAC-HPLC and SPE-HPLC methods in MDL, MQL and recovery

Analyte	Method	MDL[a] (ng/ml)	MQL[b] (ng/ml)	Variation coefficients (RSD, %)[c]	Average recovery (%)[d]		
					50ng/ml[e]	150ng/ml[f]	500ng/ml[f]
DON	IAC-HPLC	6	18	4	93.4	99.5	102.7
	SPE-HPLC	14	42	14	63.7	77.4	85.3
DOM-1	IAC-HPLC	7	21	6	85.5	91.1	87.2
	SPE-HPLC	10	30	10	68.0	77.1	82.6

[a] MDL is computed as MDL = $t_{(7,1-\alpha=0.99)} \times$ SD. $t_{(7,1-\alpha=0.99)} = 2.998$, when n=8. SD stands for the standard deviation. The MDL values were determined using spiked samples containing 50 ng/ml DON or 50 ng/ml DOM-1. $SD_{DON\text{-}IAC\text{-}HPLC}$=2.0, $SD_{DON\text{-}SPE\text{-}HPLC}$=4.6, $SD_{DOM\text{-}1\text{-}IAC\text{-}HPLC}$=2.4, $SD_{DOM\text{-}1\text{-}SPE\text{-}HPLC}$=3.5

[b] MQL is computed as MQL = MDL × 3

[c] Variation coefficients were presented as relative standard deviation (RSD, %). They were measured at concentration level 50 ng/ml (n=8)

[d] Recovery(%) = $C_{(found)}/C_{(added)} \times 100$

[e] Replication n=8

[f] Replication n=3

The developed IAC method removed most interfering matrix compounds from serum samples (Fig. 1). In contrast, serum samples after SPE clean-up contained more matrix peaks than those after IAC clean-up (Fig. 2). Most matrix peaks were eluted from 1.0 to 4.4 min. The peak high reached as high as 2,500. This high background (matrix peaks) may explain why the SPE method can not achieve detection limits as low as after IAC. Two samples were analyzed after IAC and SPE clean-up, respectively. DON at concentrations of 38.5 ng/ml (IAC) and 44.1 ng/ml (SPE) was found in sample #2. DOM-1 at 23.1 ng/ml was detected in samples #3 purified by IAC, whereas this chemical was not quantifiable after SPE clean-up.

Overall, the developed IAC-HPLC method is clearly a better choice of method for analysis of both DON and DOM-1 than the SPE-HPLC method, as it scored higher in both detection limit and recovery. Therefore, this method was chosen for determination of DON and DOM-1 in swine serum in this study.

Comparison of the developed IAC and previously published methods

The developed IAC-HPLC method had limit of detections (LOD) for DON and DOM-1 in swine blood similar to those methods previously published. The LODs (S/N=3) was estimated to be 10 ng/ml and 4 ng/ml in reports from Janes and Schuster (2001) and Valenta et al. (2003), respectively. In this study, LOD was presented by MDL, a parameter calculated using a statistical method (Glaser et al. 1981; Federal Register 1995). The MDLs for DON and DOM-1 by the IAC-HPLC method were 6 and 7 ng/ml, respectively. More significantly, this method is able to remove analytical interferences from swine serum in one quick and easy step, and to eliminate steps of extraction with organic solvent and pre-purification using SPE cartridges found in previously published methods (Janes and Schuster 2001; Valenta et al. 2003).

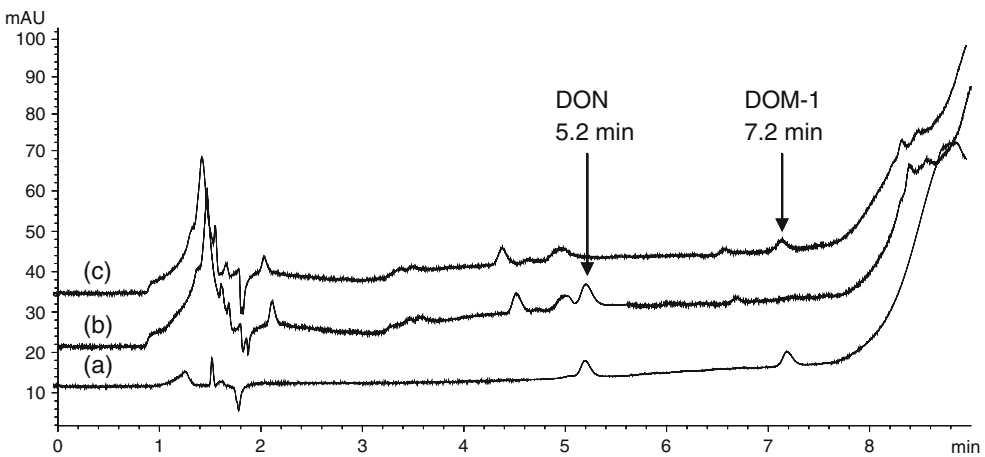

Fig. 1 HPLC chromatograms of serum samples purified by IAC. HPLC chromatograms of DON (150 ng/ml) and DOM-1 (150 ng/ml) standards (**a**), serum sample #2 (DON was quantified as 38.5 ng/ml) (**b**) and serum sample #3 (DOM-1 was quantified as 23.1 ng/ml) (**c**)

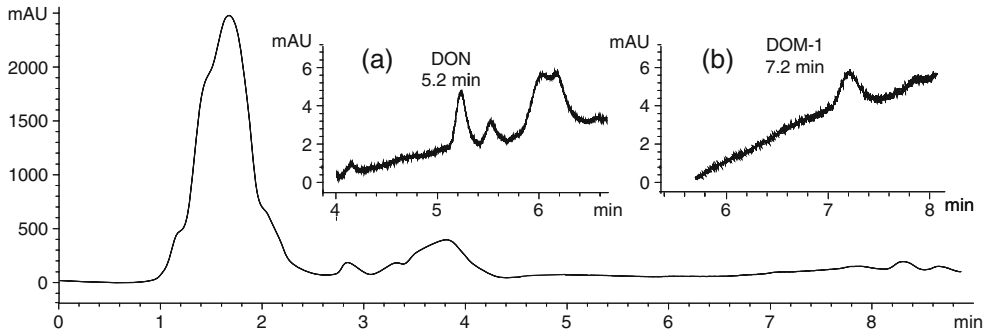

Fig. 2 HPLC chromatograms of serum samples purified by SPE. *Insert*: serum sample #2 (DON was quantified as 44.1 ng/ml) (**a**) and serum sample #3 (DOM-1 was detected, but nonquantifiable) (**b**)

DON and DOM-1 in swine serum

The IAC-HPLC method was applied to analyze DON and DOM-1 concentrations in swine serum collected from pigs in a feeding trial. The aim of this project was to determine effects of DOM-1, produced by aimed microbial transformation of DON, on swine growth performance (Li et al. 2008). Pigs were given a DOM-1 diet for 9 days during the trial. Pigs given a toxin-free corn diet served as blank control; those given a DON diet (5 mg/kg of DON) served as toxic control; pigs fed with corn fermented with the microorganisms served as a microbial control. Analytical results are shown in Table 2. No DON or DOM-1 was detected in the serum samples from pigs given a toxin-free corn diet or DON diet after microbial fermentation. Free form DON and sum DON (free form + glcuronide conjugate form) were found at 38.8±13.7 ng/ml and 49.8±14.1 ng/ml, respectively, in serum samples from pigs given a DON diet (5 mg/kg of DON). Free form DOM-1 was detected but not quantified, whereas the total DOM-1 was determined as 47.5±6.3 ng/ml in serum samples from those given a microbial-fermented DON diet, in which DON was transformed to DOM-1. These data indicated that glucuronide conjugated forms of DON and DOM-1 existed in the tested samples as metabolites of DON and DOM-1, respectively. This again demonstrated the improved performance and feasibility of the method. The improved IAC-HPLC will provide a viable tool for researchers who need to analyze DON and/or DOM-1 levels in swine blood.

Table 2 Concentrations of free form and the sum of free and conjugated forms of DON and DOM-1, respectively, in swine serum from pigs given a toxin-free corn diet, a DON diet, a detoxified-DON diet and a microbial control diet, analyzed by IAC-HPLC method

Sample	*n* [a]	DON (mean concentration ± SD[b], ng/ml)	DOM-1 (mean concentration ± SD[b], ng/ml)	The sum of free and conjugated form of DON (mean concentration ± SD[b], ng/ml)	The sum of free and conjugated form of DOM-1 (mean concentration ± SD[b], ng/ml)
Pigs given a toxin-free corn diet	6	nd[c]	nd[c]	nd[c]	nd[c]
Pigs given a DON diet (containing DON 5 μg/g; no DOM-1 was detected)	8	38.8±13.7[d]	nd[c]	49.8±14.1[e]	nd[c]
Pigs given a detoxified-DON diet (containing DOM-1; no DON was detected)	8	nd[c]	nq[f]	nd[c]	47.5±6.3[g]
Pigs given a microbial control diet	6	nd[c]	nd[c]	nd[c]	nd[c]

[a] *n* stands for replication of 6 or 8 serum samples of 6 or 8 animals per group

[b] *SD* Standard deviation of the eight replicates

[c] *nd* Nondetectable. The concentration was below the MDL

[d] The result calculated with the recovery of DON (93.4%)

[e] No data for recovery of DON-Glu are available because no DON-Glu standard was available to test. This was the result calculated with the recovery of DON (93.4%)

[f] *nq* Nonquantifiable. The result was lower than the MQL (21 ng/ml), but higher than the MDL (7 ng/ml)

[g] No data for recovery of DOM-Glu are available because no DON-Glu standard was available to test. This was the result calculated with the recovery of DOM-1 (85.5%)

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